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## Perspective

# Pax5 Maintains Cellular Identity by Repressing Gene Expression Throughout B Cell Differentiation

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## KEY WORDS

Pax5, repression, lineage commitment, Flt3, pro-B cell, Blimp-1, c-fms

## ABBREVIATIONS

Blimp1	B-lymphocyte induced maturation protein 1
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
DC	dendritic cell
EBF	early B cell factor
ELP	early lymphoid progenitor
ETP	early thymic progenitor
EPLM	early progenitor of lymphoid and myeloid developmental potential
Flt3	fms-like tyrosine kinase 3
Flt3L	fms-like tyrosine kinase 3 ligand
HSC	hematopoietic stem cell
MCSFR	macrophage colony stimulating factor receptor
MPP	multi-potent progenitor
NK	natural killer
XBP-1	X-box binding protein 1

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## ABSTRACT

The transcription factor Pax5 is required for many aspects of B-lymphopoiesis including lineage commitment, immunoglobulin rearrangement, pre-BCR signalling and mature B cell survival. Pax5 regulates B cell lineage commitment by concurrently activating B cell specific gene expression as well as suppressing the expression of genes associated with non-B cell fates. The identity of the molecular targets of Pax5-mediated gene repression is the subject of much current interest. Recent studies have documented the essential nature of the Pax5 mediated repression of the stem cell transcriptional program, as well as the silencing of lineage inappropriate gene expression, for B cell development. Surprisingly the repression of genes by Pax5 continues throughout lymphopoiesis, with the loss of Pax5 in mature B cell resulting in the reactivation of the same Pax5 targets during plasma cell differentiation. These recent insights into the mechanism of action of Pax5 in controlling B cell identity will be discussed.

## THE FUNCTION OF Pax5 IN B CELL COMMITMENT

All lineages of the blood, including B cells, are derived from a rare population in the bone marrow, the hematopoietic stem cell (HSC). HSC reside in specialized niches in the bone marrow and possess the unique capacities of self-renewal and the ability to differentiate into all the different mature blood cell types. One of the earliest differentiated progeny of the HSC is the multi-potent precursor (MPP), which is at the bifurcation of myeloid and lymphoid lineages.<sup>1</sup> MPP can further differentiate into common myeloid progenitors (CMP) or early lymphoid progenitors (ELP) but have limited ability to self-renew. ELP themselves give rise to the early thymic T cell progenitor (ETP) or the common lymphoid progenitor (CLP).<sup>2</sup> CLPs have B cell, natural killer (NK) cell, dendritic cell (DC), but limited T cell, potential. The first B cell progenitor arises from the CLP in the bone marrow and is variously termed the pre-pro B cell, fraction A, or CLP-2 and can be identified by the expression of the B cell specific marker B220.<sup>3</sup>

One of the earliest regulatory events associated with lymphoid development is the expression of the fms-like tyrosine kinase 3 (Flt3) on a subset of MPP and CLP.<sup>1,4</sup> Flt3 and Flt3L-deficient mice display a marked decrease in B cell progenitors and their HSCs are impaired in their ability to differentiate into myeloid and lymphoid cells.<sup>5-7</sup> Flt3 ligand (Flt3L) also plays an important role in the generation of DCs.<sup>8</sup> The signalling pathway through which Flt3 selectively favours the generation of B cell progenitors over other lineages such as DC is currently unknown. The other essential growth factor for early lymphopoiesis is IL-7.<sup>9-11</sup> The IL-7 receptor (IL-7R) is composed of the common  $\gamma$  chain and the IL-7R $\alpha$  chain, which is also a component of the thymic stromal derived lymphopoietin receptor (TSLP-R).<sup>12</sup> In keeping with its dual role, IL-7R $\alpha$  deficient mice have a more pronounced B cell deficiency than *common  $\gamma$  chain*<sup>-/-</sup> mice which have an intact TSLP-R.<sup>13,14</sup> Interestingly, mice doubly deficient for Flt3L (or Flt3) and IL-7R $\alpha$  lack any B cells demonstrating that together IL-7 and Flt3L are essential for virtually all B cell development.<sup>15,16</sup>

Specification and commitment of B lymphopoiesis also critically depends on the expression of a number of transcription factors: Ikaros, PU.1, E2A, Early B cell factor (EBF) and Pax5.<sup>17,18</sup> While the exact functions of Ikaros and PU.1 in lymphoid progenitors is still unclear, Ikaros deficient mice lack *Flt3* expression,<sup>19,20</sup> whereas PU.1 is known to promote the initiation of *IL-7R $\alpha$* <sup>21</sup> and *EBF* expression.<sup>22,23</sup> Once defined B lymphoid progenitors are formed, the transcription factors E2A and EBF act in concert to initiate the expression of B cell specific genes in the earliest B cell progenitors.<sup>24</sup> Despite

initiating the expression of some B cell specific genes, these two factors do not induce commitment to the B cell lineage, a process that depends on Pax5.

Pax5 is a multifunctional transcriptional regulator that is expressed throughout the B cell lineage, from the pro-B cell stage until its down-regulation in plasma cells.<sup>17,18</sup> In the absence of Pax5, B cell development is arrested at the early pro-B cell (or pre-BI) stage of differentiation, characterised by the expression of many B cell specific transcripts and *D-J<sub>H</sub>* rearrangements at the *IgH* locus.<sup>25</sup> Intriguingly, while being unable to differentiate into mature B cells, *Pax5*<sup>-/-</sup> pro-B cells can be cultivated indefinitely in vitro in the presence of IL-7 and stroma. Most surprisingly, these pro-B cells are not committed to the B cell lineage and are capable of differentiating into a broad spectrum of hematopoietic cell types.<sup>26,27</sup> The restoration of Pax5 expression in deficient cells suppresses this multi-lineage potential, whereas the conditional inactivation of Pax5 in pro-B cells reverts lineage commitment and again generates multi-potent cells.<sup>28</sup> A similar capacity was subsequently reported for *E2A*<sup>-/-</sup> lymphoid progenitors, a finding in keeping with the fact that these cells lack *Pax5* expression.<sup>29</sup> Recently a number of studies have sought to address the mechanism by which Pax5 initially promotes and then maintains lineage commitment. These recent insights into Pax5 function will be discussed in detail below.

## Pax5 REPRESSES NON-B CELL SPECIFIC GENES TO ALLOW B CELL COMMITMENT

Gene expression analysis of HSCs and various uncommitted hematopoietic progenitors has shown that multi-potent cells maintain the concurrent expression of genes associated with different lineages. This phenomenon is termed "lineage priming" and it is proposed that stem cells or uncommitted progenitors maintain a relatively open chromatin configuration which often results in low-level expression of genes reflective of multiple incompatible lineage fates.<sup>30,31</sup> By this model, differentiation and ultimately lineage commitment is expected to result in the progressive repression of this lineage-promiscuous gene expression until a stable transcriptional profile is reached. As Pax5 has the capacity to both activate and repress genes, it was hypothesized that this transcription factor may promote B cell lineage commitment by repressing the expression of lineage inappropriate genes. Indeed multi-potent *Pax5*<sup>-/-</sup> pro-B cells were shown to maintain promiscuous expression of myeloid, erythroid, T and NK cell genes, despite expressing multiple B cell specific genes.<sup>26</sup> A striking feature of this lineage-priming was the expression of cell surface receptors, such as *c-fms* (macrophage colony stimulating factor receptor (*M-CSFR*)) or *Notch1*, which allowed Pax5-deficient pro-B cells to respond to external signals and differentiate into macrophages and T cells, respectively (Fig. 1).<sup>26,32,33</sup> Reintroduction of Pax5 expression into these cells leads to the repression of these non-B cell committed genes thus abolishing multi-lineage potential, whereas the inactivation of Pax5 in committed pro-B cells results in the re-expression of these genes.<sup>28</sup> These data support a model whereby Pax5 controls B cell fate both by activating B cell specific target genes promoting B-lymphopoiesis and simultaneously repressing the non-B cell fate. Recent studies have now addressed three previously

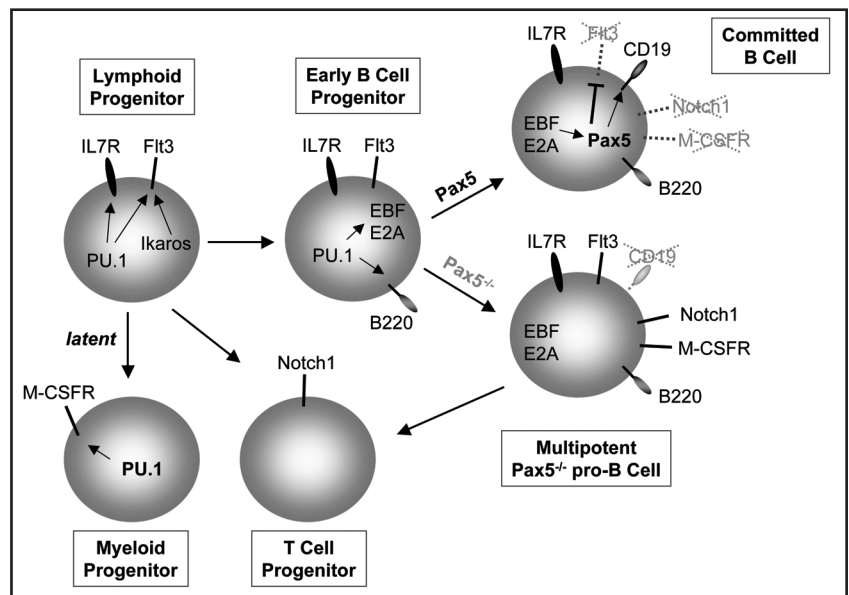


Figure 1. Pax5-mediated gene repression is essential for B cell lineage commitment. The key transcription factors and growth factor receptors are shown. Lymphoid progenitors are capable of "latent" myeloid differentiation in certain circumstances. ↓ Indicates positive interactions and ⊥ indicates repression. Genes dependent on Pax5 are indicated by a hatched line. In the absence of Pax5, pro-B cells remain multipotent and can differentiate into myeloid and T cells.

unresolved issues: firstly, what is the crucial target(s) of Pax5 repression in lineage commitment; secondly, how many genes are repressed by Pax5 in early and late B cell differentiation; and finally, how mechanistically does Pax5 achieve these functions.

## REPRESSION OF FLT3 BY Pax5 IS CRUCIAL FOR B CELL COMMITMENT

As Pax5 expression directly results in B cell commitment, we hypothesized that one strategy by which it promotes this process is to repress the expression of genes required to maintain stem cell fate. As mentioned above, expression of Flt3 on early hematopoietic progenitors is important for multi-lineage potency and the generation of B cell progenitors. Interestingly, B cells down-regulate *Flt3* expression during B cell commitment.<sup>34</sup> Thus *Flt3* represented an attractive candidate for Pax5 repression. Indeed, we have recently reported that, in contrast to the great majority of wild type pro-B cells (B220<sup>+</sup>ckit<sup>+</sup>), *Pax5*<sup>-/-</sup> pro-B cells uniformly express Flt3 on their cell surface, suggesting that Pax5 may be involved in directly repressing *Flt3* expression.<sup>35</sup> In agreement with this prediction, the restoration of Pax5 in the mutant pro-B cells led to the rapid silencing of *Flt3*. Promoter analysis indicated that this repression is likely to be direct as we identified two Pax5 binding sites within the *Flt3* promoter region that are occupied in vivo. To test the importance of the Pax5-mediated repression of *Flt3* in B cell commitment we used a retroviral vector to ectopically express *Flt3* throughout the hematopoietic system and analyzed the consequences for B cell development.<sup>35</sup> Strikingly, enforced *Flt3* expression resulted in a pronounced decrease in all stages of bone marrow B-lymphopoiesis in transplanted mice while thymocyte and myeloid differentiation was relatively unaffected. This impaired B cell differentiation was also observed in vitro after the transduction of stem cell enriched populations and was not the result of Flt3-induced cell death.

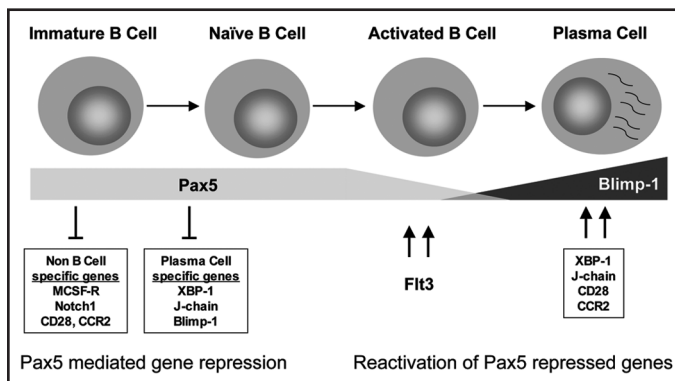


Figure 2. The function of Pax5 and Blimp-1 in late B cell differentiation. The stages of cellular differentiation from a resting B cell to an antibody secreting plasma cell are indicated, as are the relative levels of Pax5 and Blimp-1 expression. Pax5 represses non-B cell and plasma cell genes in immature and naïve B cells. Upon activation, Pax5 function is inhibited and the expression of Pax5-repressed genes such as *Flt3* and ultimately *Blimp-1* are induced. A number of Pax5-repressed genes are then important for plasma cell function.

The importance of Pax5 mediated repression of *Flt3* has recently received independent confirmation by the report that high doses of injected *Flt3L* results in a pronounced increase in the frequency of a population of the wild type progenitor cells termed the EPLM (early progenitor of lymphoid and myeloid developmental potential).<sup>36</sup> EPLMs appear to have phenotypic and developmental capacities very similar to *Pax5*<sup>-/-</sup> pro B cells, demonstrating that the developmental pathway characterized in the absence of Pax5 also occurs in a transient manner in wild type cells.<sup>37</sup> Importantly *Flt3L* exposure also resulted in a marked decrease in B lymphocyte progenitors, whereas myeloid and T cells were again unaffected.<sup>36</sup> As in vitro studies have clearly shown that high levels of *Flt3L* can induce DC potential in a variety of progenitors,<sup>8,38</sup> we propose that the inability of *Pax5*<sup>-/-</sup> pro B cells to silence *Flt3* expression enables these multi-potent cells to be influenced by other signals and thus allow the commitment to alternate lineages, such as DC, at the expense of B cells.

In summary, this data demonstrates how factors such as E2A and EBF that induce Pax5 expression and ultimately lineage commitment can at the same time result in the repression of *Flt3*, promoting B lineage commitment by a negative feedback mechanism (Fig. 1).

## CONTINUOUS Pax5 EXPRESSION IS ESSENTIAL FOR THE REPRESSION OF LINEAGE INAPPROPRIATE GENES

One of the limitations to fully understanding the function(s) of Pax5 in B cells was the fact that, until recently, few of the genes repressed by Pax5 were known. To overcome this limitation, Delogu et al have utilised a global transcriptional profiling approach to identify and validate 110 Pax5 repressed genes in pro-B and mature B cells.<sup>39</sup> Collectively these genes have important functions in a broad range of biological activities including cell-cell communication, adhesion, migration, nuclear processes and cellular metabolism. In strong support of the lineage-priming model, 68% of these repressed genes are also expressed in erythro-myeloid or T cell lineages and a substantial fraction of these are found in multi-potent progenitor fractions such as HSC, CMP and CLP.

The Pax5-mediated repression of these genes is not only critical for B cell commitment but is also important in maintaining B cell identity and function of committed pro-B cells, as inactivation of

Pax5 in pro-B cells induced the rapid expression of a large number of previously repressed genes.<sup>39</sup> This capability formally demonstrates that the ectopic expression of these genes in *Pax5*<sup>-/-</sup> pro-B cells was not a result of incomplete lineage specification, but an active repression process dependent on Pax5. Furthermore, this repression was still essential in mature B cells as the deletion of Pax5 resulted in the re-expression of many of the formerly silent genes, including *Flt3* (Fig. 2).

In contrast to early B cell development where Pax5 expression is maintained constitutively, Pax5 is normally down-regulated during the terminal differentiation of activated B cells into antibody secreting plasma cells.<sup>40</sup> Delogu et al therefore addressed whether the Pax5-mediated repression of non-B cell specific genes is also alleviated after the physiological down-regulation of Pax5 during plasma cell differentiation. Indeed, many repressed genes were reexpressed in plasma cells due to the absence of Pax5 expression (Fig. 2). This re-expression was not an irrelevant consequence of the loss of Pax5 as two proteins, the coreceptor CD28 and the chemokine receptor CCR2, were shown to be important for plasma cell function in an immune response.<sup>39</sup>

Interestingly, the conditional deletion of *Pax5* in mature B cells also resulted in the expression of *Blimp-1* (B lymphocyte induced maturation protein, also termed *Prdm1*), an essential transcriptional regulator of plasma cell development,<sup>41</sup> and the *J chain*, a known Pax5-repressed gene required for efficient antibody secretion.<sup>42</sup> Using chicken DT40 cells as an independent model, Nera et al also noted the activation of the plasmacytic transcription factors Blimp-1 and X-box binding protein 1 (XBP-1)<sup>43</sup> and increased IgM secretion after *Pax5* inactivation.<sup>44</sup> Together these studies suggest that Pax5 normally functions in mature B cells to repress the plasma cell pathway through suppressing, directly or indirectly, *Blimp-1* expression. As Blimp-1 is known to directly repress *Pax5* expression,<sup>45</sup> it was proposed that the mutually antagonistic functions of these two pivotal transcription factors controls B cell terminal differentiation. However, recent experiments from our laboratory suggest that this interaction is more complex than anticipated, as the derepression of Pax5 target genes occurs in the absence of Blimp-1, suggesting that other factors are also essential in controlling Pax5 activity during B cell terminal differentiation (Fig. 2 and Kallies et al., submitted).

## Pax5 CAN REPRESS GENE EXPRESSION BY DIRECTLY INTERFERING WITH THE TRANSCRIPTIONAL MACHINERY

An important question that arises as a consequence of the observations outlined above is, how does Pax5 function on a molecular level to achieve the repression of this diverse range of genes? While in vitro biochemical studies have shown that Pax5 can directly repress gene transcription by recruiting members of the groucho family of corepressors, this interaction has not been demonstrated to be functionally relevant on endogenous Pax5 target genes.<sup>46</sup> Moreover, while Pax5 represses the expression of a large number of myeloid genes in B cells, the enforced over-expression of Pax5 in the myeloid lineage has no pronounced effect.<sup>32,47</sup> A recent study using the *c-fms* locus (encoding the MCSF-R) as a model Pax5-repressed gene has shed some light on this issue.<sup>48</sup> Interestingly, the *c-fms* locus is in an active or open chromatin configuration in *Pax5*<sup>-/-</sup> pro-B cells whereas upon re-expression using an inducible system Pax5 is rapidly recruited to the *c-fms* promoter. This recruitment results in the immediate cessation of transcription as measured by the selective loss of RNA polymerase II localization. Pax5 induction



also leads to a delayed decrease in the binding of PU.1, an essential regulator of *c-fms* expression in macrophages.<sup>48</sup> DNA binding analysis indicated that Pax5 bound to the *c-fms* promoter directly adjacent to a PU.1 site, and that Pax5 inhibited PU.1 function but not binding. The model to emerge from these studies proposes that in cells expressing high levels of *c-fms*, such as macrophages, the full complement of transcription factors are present and drive maximal transcription. In this scenario, ectopic Pax5 has minimal impact and explains to lack of a functional consequence of Pax5 overexpression in myeloid cells. However, in lymphoid progenitors, the *c-fms* locus is in an accessible or primed chromatin configuration but lacks the expression and/or concentration of essential myeloid factors such as PU.1.<sup>49</sup> This configuration leads to low-level transcription that is repressed by Pax5 throughout B-lymphopoiesis. This concept provides a molecular rationale for lineage priming and indicates how the antagonistic interactions of a few master-regulatory transcription factors can control many important cell fate and differentiation decisions (Fig. 1). It remains to be determined whether the specifics of this model can be more broadly generalized as a mechanism to explain Pax5-mediated gene repression.

## CONCLUSION

The control of B cell development by Pax5 represents one of the leading model systems to investigate the transcriptional regulation of hematopoietic cell differentiation choices. Interestingly, Pax5 controls many aspects of B cell biology including lineage commitment, antigen receptor rearrangement and signalling, and the control of terminal differentiation. Obtaining a cellular and molecular understanding of how one factor can achieve all these disparate functions has proven a difficult challenge. However the recent cataloguing of Pax5 repressed genes as well as the development of plausible cellular and molecular models to understand Pax5-mediated gene repression are shedding light on these processes. These advances have highlighted the synergistic benefit of pursuing a variety of approaches, including global genomic, biochemical and precise gene-by-gene strategies, in understanding the complex interplay between gene regulation and cellular differentiation.

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